

## **REMARKS/ARGUMENTS**

### **Interview Summary**

A telephonic interview was conducted on or about Feb. 11, 2008 with Examiner Zeman. Enablement of the scope of the method claims was discussed, regarding the term “antigen presenting cell” and the diseases or disorders being treated. This supplemental response is filed in response.

### **Claim Amendments**

The claims have been amended to better clarify the invention. Support for the amendments can be found throughout the application as filed, including in the Examples and as shown in the drawings. No new matter is added.

Support for the amendments reciting that the antigen-presenting cell is a dendritic cell can be found in the Examples and in claims 2, 6, 9, 12, 15, 19 and 28-29 as filed.

Support for the amendments reciting that method of increasing the expression of interleukin 10 (IL10) in a mammal can be found in paragraph 0002 (“The present invention also provides a process for preparing novel synthetic SPAs that can be used to induce the activity of T regulatory cells and the expression of interleukin 10 (IL10) in humans and other animals ...”), in paragraphs 0010, 0047 (and Figure 3), 0079, 0081, 0091, 0093, 0105, and in Examples 2 and 6 (see page 68-70 and Table 6: up-regulation of IL10 was “[a]lso seen with Compound 15 @ 100 ug/ml”).

Support for methods of treating or preventing abscess and sepsis using compound 15 (new claims 34-40 and 42-44) is found throughout the application as filed, including in Example 13 at pages 70-72, where administration of CP1 and Compound 15 were shown to decrease the percent of animals with abscesses in a dose-dependent fashion. Additionally, it was stated that “nor do these animals display symptoms of sepsis” (page 71, lines 29-30). As discussed during the telephonic interview, the rat model presented in Example 13 is also used as a model for sepsis, and therefore this example also provides a working example of treating or preventing sepsis. A reference demonstrating use of this rat model as a model for sepsis is attached as Exhibit A.

Support for methods of treating or preventing post-surgical adhesions using compound 15 (new claims 34-40 and 42-44) is found throughout the application as filed, including in Example 15 at pages 72-75 (sic, now Example 14 as amended), where administration of CP1 and Compound 15 were shown to decrease the mean adhesion score in an animal model of post-surgical adhesions.

Support for new claim 41 can be found throughout the application, for example at page 34 line 24-page 35 line 25, and at page 33 lines 26-27, and in Example 1 regarding the synthesis and purification of Compound 15.

### **CONCLUSION**

It is respectfully submitted that the above-identified application is now in condition for favourable consideration of all the claims, and prompt allowance of these claims is respectfully requested. Should the Examiner believe that anything further is desirable in order to place the application in better condition for allowance, the Examiner is invited to contact the applicant's undersigned attorney at the telephone number listed below.

Respectfully submitted

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# Protection against Lethal Intra-abdominal Sepsis by 1-(3-dimethylaminopropyl)-3-ethylurea

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Sodium hyaluronate-carboxymethylcellulose (HA/CMC) formulations are gels that effectively reduce post-operative adhesions in both animals and humans, when placed in the peritoneal or pelvic cavities concomitant with surgical manipulation. However, it has been suggested that the use of these products may increase the risk of peritoneal infection after contamination with intestinal contents during surgery. Using the rat intra-abdominal sepsis model, we found that administration of HA/CMC gels before bacterial challenge did not increase mortality but did significantly protect rats against lethal infection. This effect was dose and time dependent. Protection was conferred not by the HA/CMC gels themselves but by 1-(3-dimethylaminopropyl)-3-ethylurea (EDU), a small molecule released from the gel complex under physiologic conditions. Our results suggest that the protective effect exhibited by EDU is related to down-regulation of T cell-dependent responses and suppression of the proinflammatory-cytokine cascade associated with mortality during the early phase of disease.

The development of adhesions is a major cause of morbidity in patients who have undergone abdominal or gynecological surgery [1]. Hyaluronate-based gels have been shown to reduce adhesions in both animals and humans when placed in the peritoneal or pelvic cavity [1–3]. However, because the use of these products requires surgical manipulation, there is the risk of bowel disruption or accidental contamination of the peritoneal cavity with intestinal contents containing a large number and variety of microorganisms, which can provoke fatal sepsis [4]. Intra-abdominal sepsis is characterized by a massive leukocyte infiltration into the peritoneum, followed by an overwhelming systemic response via the voluminous production of inflammatory mediators, including cytokines, which results in shock or multiple organ failure [5].

The rat model of intra-abdominal sepsis has been used extensively to study the roles of various micro-

organisms during the infectious process and to test the therapeutic efficacy of a variety of antimicrobial agents. The predictive value of the rat model for human sepsis has been well documented [6–10]. In previous investigations, this model was selected to investigate whether the placement of adhesion-reduction materials could affect the progression of bacterial contamination in the abdominal cavity; Tzianabos et al. [11] have shown that some adhesion reduction products, such as HA (sodium hyaluronate)/CMC (carboxymethylcellulose)/NAU (*N*-acyl urea) gels, placed over the intestines of animals at the time of bacterial challenge greatly increase lethality. However, other products, such as HA/CMC chemically modified with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Seprafilm; Genzyme), reduce the incidence of adhesions as intended and also do not increase mortality, do not alter the course of disease, and may, in fact, decrease lethality.

The rat model of intra-abdominal sepsis also has been used widely to explore the host immune response during intra-abdominal infections. It has been extensively reported that a purified polysaccharide from *Bacteroides fragilis* (PS A), administered shortly before microbial challenge, protects against development of abscesses in this model, by modulation of a T cell-dependent host immune response that leads to abscess

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formation [12–15]. Other polysaccharides with immunomodulatory characteristics, such as soluble  $\beta$ -glucan, have been shown to suppress mortality when they are administered before infectious challenge; the protective mechanism involves up-regulation of prostaglandin E<sub>2</sub>, which, in turn, down-regulates the early inflammatory process [16]. In addition, HA has been reported to act as an immunomodulator in this model system [2, 11]. On the basis of these observations, we hypothesized that HA/CMC gels have, along with their capacity to reduce adhesions, some immunomodulatory properties. To test our hypothesis, we explored the effect on mortality of administration of HA/CMC gels by different routes and at different times in the rat intra-abdominal sepsis model.

## MATERIALS AND METHODS

**Rat intra-abdominal sepsis model.** Male VAF Wistar rats (175–200 g) purchased from Charles River Laboratories were used for all in vivo experiments. Rats were housed according to Brigham and Women's Hospital and Harvard Medical School guidelines for care and use of animals and received sterile water and food ad libitum. Surgery was performed as described elsewhere [7, 9]. Rats were anesthetized with a single intraperitoneal (ip) injection of sodium pentobarbital (7.5 mg/rat; Abbott Laboratories), the abdomen was shaved and cleaned with iodine, and a 0.5-cm incision was made through the anterior abdominal wall. After 0.5 mL of inoculum was placed in the abdominal cavity, the incision was closed with silk suture. Mortality within 4 h of surgical implantation of the inoculum was considered to be an anesthetic-related death, and rats that died in that way were not included in the final results for each experiment.

**Reagents.** HA/CMC/EDC gel, CMC gel, CMC/EDC gel, HA/EDC gel, and 1-(3-dimethylaminopropyl)-3-ethylurea (EDU) were provided by Genzyme as endotoxin-free solutions. Intergel (0.5% ferric ion cross-linked hyaluronate) was purchased from Lifecore Biomedical. All dilutions were prepared in sterile, pyrogen-free saline solution.

**Experimental design.** Groups of 10–20 rats were administered varying doses of sterile HA/CMC gel, components of the gels, EDU, or saline solution, by intramuscular (im) injection at various times before and after surgical implantation of the bacterial inoculum. In some cases, gels were placed in the peritoneal cavity of rats. In further experiments, osmotic pumps (Alzet) loaded with 100  $\mu$ L of EDU (100 mg/mL) were used, at a release rate of 0.92  $\mu$ L/h. After injection with sodium pentobarbital, pumps were implanted subcutaneously into the posterior scapulae area of rats 1 day before challenge. The incision was closed with silk suture, and rats were returned to their cages until bacterial challenge.

An inoculum of *Escherichia coli* ( $1 \times 10^8$  cfu/mL) in peptone yeast glucose broth containing 10% (vol/vol) sterile cecal contents

was used for experiments in which mortality was the end point. After surgery, rats were observed twice per day until the completion of the experiment. All rats that died of the infection did so within 48 h after challenge. For those experiments involving the measurement of cytokines and proliferation of T lymphocytes, a sublethal dose of *E. coli* ( $7 \times 10^7$  cfu/mL) in peptone yeast glucose broth containing 10% (vol/vol) sterile cecal contents was used. At various times after surgery, a minimum of 5 rats/group were killed. Spleens were aseptically removed, and half of each spleen was weighed and homogenized in PBS containing 0.5% protease inhibitor cocktail (Sigma Chemical) in a tissue homogenizer (OMNI International). Homogenates were frozen at  $-70^\circ\text{C}$  for  $\sim 30$  min, thawed at  $37^\circ\text{C}$ , and centrifuged at 3000 g for 15 min. Supernatants were collected and stored at  $-70^\circ\text{C}$  for assessment of cytokine levels. Splenocytes were obtained by dispersion of the other half of each spleen through a sterile 100- $\mu$ m plastic mesh (Falcon; Becton Dickinson Labware) in RPMI 1640 medium (Gibco BRL Life Technologies) with 5% fetal calf serum (FCS; Gibco BRL Life Technologies). Cells were fractionated by Lymphoprep gradient (Nycomed Pharma AS) at 800 g for 30 min at  $20^\circ\text{C}$ . The mononuclear layer (T lymphocytes, B lymphocytes, and other mononuclear cells) was removed and washed 3 times in medium.

**Cell culture.** Splenic mononuclear fractions obtained from challenged rats, as described above, were cultured in RPMI 1640 medium with 5% FCS containing antibiotics, in 96-well round-bottom plates (Falcon; Becton Dickinson Labware) in triplicate at  $3 \times 10^5$  mononuclear cells (MNCs)/well. Cells were stimulated with 3  $\mu$ g/mL Concanavalin A (ConA; Sigma Chemical).

For in vitro studies, spleens were aseptically removed from killed male Lewis rats (175–200 g), and splenic mononuclear fractions were obtained as described above. Some of these cells were irradiated (2500 rad) for use as antigen-presenting cells (APCs). T cell purification was performed by depletion of B cells and monocytes by passage over nylon wool columns (Polysciences). Nylon-passed cells were  $>98\%$  CD3<sup>+</sup>, as shown by fluorescence-activated cell sorter analysis. Purified T cells and APCs were cultured in RPMI 1640 medium with 5% FCS containing antibiotics, in 96-well round-bottom plates, in triplicate at  $1 \times 10^5$  T cells/well and  $1 \times 10^5$  APCs/well, respectively. Cells were stimulated with 3  $\mu$ g/mL ConA, and different concentrations of EDU or cyclosporin A (CsA; Sigma Chemical) were added to the cultures. On day 2 or 4, a 50- $\mu$ L aliquot of culture was removed from each experimental well and assayed for lactate dehydrogenase (LDH) by use of the CytoTox96 Cytotoxicity Assay (Promega), according to the manufacturer's instructions. LDH release was used as an indicator of cell integrity. Supernatants were also assayed for cytokine production.

Peritoneal macrophages were purified from sterile peritoneal lavage obtained from killed male Lewis rats (175–200 g). After 20 mL of saline was injected slowly into the peritoneal cavity,

the abdomen was gently massaged, and saline was carefully removed. Cells were plated in MEM with 10% FCS (Gibco BRL Life Technologies) at  $1 \times 10^6$  cells/well in a 12-well tissue culture plate. After incubation for 90 min at 37°C, the nonadherent cells were removed by washing the wells 3 times with sterile PBS. Under these conditions, the adherent monolayer consisted of ~90% macrophages. Cells were stimulated with 1 µg/mL *E. coli* lipopolysaccharide (LPS; Sigma Chemical) and 10 ng/mL interferon (IFN)-γ (BioSource International), and different concentrations of EDU were added to the cultures. Plates then were incubated overnight at 37°C in 5% CO<sub>2</sub>, and supernatants were assayed for cytokine production.

**T cell proliferation measurement.** Splenic MNCs or purified T cell cultures were pulsed with 0.02 µCi of [<sup>3</sup>H]thymidine/well (NEN; Life Science Products) on day 1 or 3 of culture for 16 h. Plates then were incubated overnight at 37°C in 5% CO<sub>2</sub> and harvested on day 2 or 4, to collect DNA onto a paper filter, according to the manufacturer's instructions (Perkin Elmer Life Science). The filter was placed in a bag filled with scintillation liquid (Betaplate Scint; Perkin Elmer) and transferred to a β-scintillation counter (Wallac Microbeta Trilux; Perkin Elmer). Data were expressed as counts per minute of triplicate experimental cultures. For all proliferation experiments, data represent typical results for at least 3 different experiments.

**Cytokine measurements.** Rat-specific enzyme immunoassays for tumor necrosis factor (TNF)-α, interleukin (IL)-2, IFN-γ, IL-1, monocyte chemotactic protein (MCP)-1, IL-10, IL-6, and IL-4 were performed according to the manufacturer's instructions (BioSource International). Samples were run in triplicate, and the concentration was calculated on the basis of a standard curve established with the mean values of standard concentrations run in triplicate. The lower detection limit was 3–8 pg/mL.

**Statistical analysis.** Mortality rates between groups were compared by use of Fisher's exact test. Cytokine levels were compared by use of the Mann-Whitney *U* test (GrafPad; Instat Software). In all cases, data represent the mean ± SD of at least 3 separate experiments. *P* < .05 was considered to be significant.

## RESULTS

**Protection against mortality by HA/CMC gel.** Previous work by Tzianabos et al. [11] using the rat intra-abdominal sepsis model showed that placement of HA/CMC/EDC over the intestines of rats at the time of bacterial challenge did not increase lethality. Moreover, although the difference was not statistically significant, mortality in treated rats was lower than that in control rats [11]. To address whether HA/CMC gels protect rats from lethal sepsis, we first treated the rats by different routes and at different intervals, using the intra-abdominal sepsis model (table 1). Treatment at the time of challenge with 2

mL of HA/CMC, by either ip placement or im injection, did not confer protection against mortality, compared with mortality in control rats. However, similar treatment 24 h before bacterial challenge significantly decreased mortality. Protection was observed in both ip- and im-treated rats, a finding that suggests that gels might have a systemic effect that results in the reduction of mortality. To characterize the observed protection further, groups of 10 rats were administered a single dose of 2, 1, 0.5, 0.1, or 0.05 mL of HA/CMC gel or 2 mL of saline im 24 h before bacterial challenge (figure 1). Mortality in the saline-treated rats was 68% ± 14%. Rats receiving 0.1 or 0.05 mL of gel had mortality rates similar to those of control rats. However, rats treated with 0.5–2.0 mL of gel showed significant dose-dependent reduction in mortality.

**Effect of gel composition on protection.** HA/CMC gel was composed of 5% (wt/wt) chemically derivatized HA and CMC in buffered saline. The reaction of both polysaccharides with EDC and subsequent rearrangement to *N*-acylurea renders a proportion of the carboxylate groups cationic. An ionic association between the negatively charged carboxylate groups and the positively charged *N*-acylurea groups slows down gel resorption from the peritoneum. Gels then are hydrolyzed in vivo, turning into EDU and free HA and CMC. To determine the specific components of HA/CMC gel responsible for protection in the rat model, HA, CMC, CMC/EDC, HA/EDC, and EDU were administered at a dose of 0.5 mL im 24 h before surgical implantation of the inoculum. The results shown in table 2 indicate that treatment with the HA/CMC gel cross-linked with EDC, CMC/EDC, HA/EDC, and EDU was protective against lethal infection, compared with treatment with saline, HA, or CMC alone. EDU was the common constituent of the gel combinations that conferred protection.

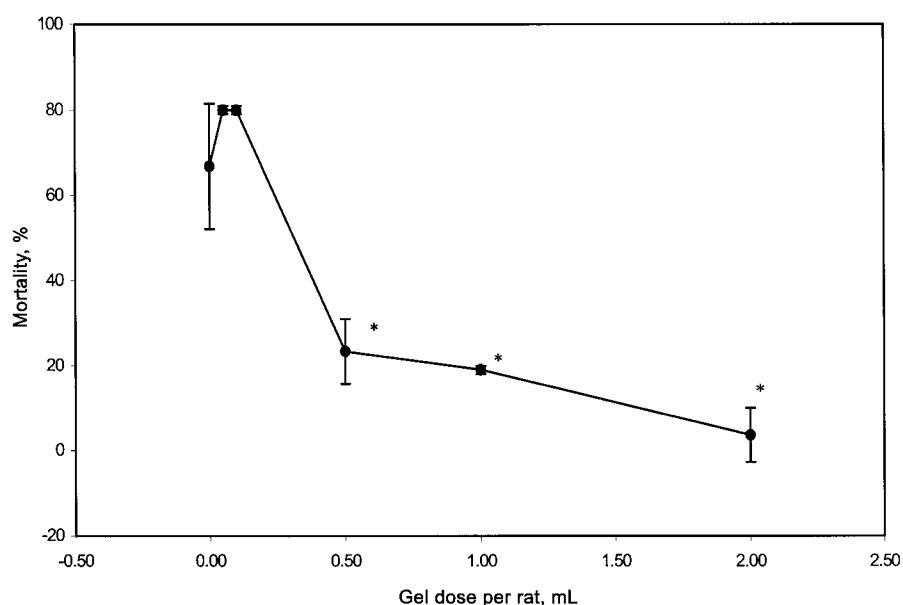
**Protection against mortality by EDU.** To verify whether EDU confers protection, rats were treated with 0.5 mL of EDU

**Table 1. Effect of time and route of administration on protection against mortality by sodium hyaluronate (HA)-carboxymethylcellulose (CMC) gels.**

Method of administration, time before challenge	Control	HA/CMC gel
Intraperitoneal		
0 h	14/19 (74)	31/38 (81)
24 h	14/20 (70)	0/20 (0) <sup>a</sup>
Intramuscular		
0 h	25/33 (75)	10/16 (62)
24 h	25/33 (75)	2/32 (6) <sup>a</sup>

**NOTE.** Data are no. rats deaths/total no. of rats (% mortality). Rats received 2 mL of HA/CMC gel or saline by placement in the peritoneal cavity (intraperitoneal) or by intramuscular injection 24 h before or at the time of challenge with  $1 \times 10^9$  cfu of *Escherichia coli* and sterile cecal contents.

<sup>a</sup> *P* < .0001, vs. control.



**Figure 1.** Protection against mortality by sodium hyaluronate–carboxymethylcellulose (HA/CMC) gels. Rats received different doses of HA/CMC gel or saline by intramuscular injection 24 h before challenge with  $1 \times 10^9$  cfu of *Escherichia coli* and sterile cecal contents. Data are mean  $\pm$  SD of at least 3 separate experiments. \* $P < .001$ .

(4 mg/mL) at various times before and after bacterial challenge. As shown in table 3, EDU did not confer protection when administered either at the time of or slightly before challenge. However, a single injection of EDU 24 h before challenge resulted in a statistically significant reduction in mortality. Administration of EDU at 24 and 4 h before and again at 24 h after challenge resulted in complete protection against the lethal effects of bacterial challenge. Hence, bioavailability of EDU over time seemed to be crucial to protection in this animal model. This hypothesis was tested by administration of EDU by surgically implanted osmotic pumps that slowly release this compound beginning 24 h before bacterial challenge. These pumps release EDU at 100  $\mu$ g/h over a 3-day period (2.4 mg/day). In this experiment, EDU-treated rats had a mortality rate of  $18\% \pm 9\%$ , compared with  $51\% \pm 11\%$  in the saline-treated control rats ( $P < .01$ ).

Treatment with EDU at subtherapeutic doses appeared to alter the time course of deaths. In the control group, ~90% of the rats that died from the infection did so within the first 24 h after surgery, whereas, in the treated groups, that figure decreased to 70% (data not shown). In both groups, all rats that died from the infection did so within 48 h after challenge.

#### Effect of EDU on cytokine production after challenge.

Cytokine production after EDU treatment was assessed in rats challenged with a sublethal dose of *E. coli*. Levels of TNF- $\alpha$  were significantly lower in these rats 4 h after challenge than were levels in those receiving saline. TNF- $\alpha$  levels quickly decreased in both groups after this time (figure 2). Like TNF- $\alpha$  levels, spleen IL-2 concentrations in control rats were  $1.7 \pm$

0.1 times greater than those in the EDU-treated rats at 4 h after challenge ( $P < .01$ ). Although concentrations of IFN- $\gamma$ , IL-1, IL-10, MCP-1 (figure 2), and IL-6 (data not shown) in the spleen increased after bacterial challenge, there was no significant difference between control and treated rats. IL-4 levels were below detection limits in all the samples.

#### Effect of EDU in proliferation of T lymphocytes in vivo.

T cell function was investigated in rats during intra-abdominal sepsis. Rats were treated with 0.5 mL of EDU (4 mg/mL) every 12 h for 2 days before and 2 days after bacterial challenge. A

**Table 2.** Protection against mortality by components of sodium hyaluronate (HA)–carboxymethylcellulose (CMC) gel.

Treatment	Mortality, %
Saline	66 $\pm$ 9.1
HA/CMC	20 $\pm$ 7.0 <sup>a</sup>
HA	79 $\pm$ 1.0
CMC	68 $\pm$ 1.0
HA/EDC	20 $\pm$ 1.0 <sup>a</sup>
CMC/EDC	26 $\pm$ 1.0 <sup>a</sup>
EDU	20 $\pm$ 1.0 <sup>a</sup>

**NOTE.** Data are mean  $\pm$  SD of at least 3 separate experiments. Rats received 0.5 mL of HA/CMC gel, HA, CMC, HA/1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), CMC/EDC, 1-(3-dimethylaminopropyl)-3-ethylurea succinate (4 mg/mL), or saline by intramuscular injection 24 h before challenge with  $1 \times 10^9$  cfu of *Escherichia coli* and sterile cecal contents.

<sup>a</sup>  $P < .001$ , vs. control.

**Table 3. Effect of time of administration of 1-(3-dimethylaminopropyl)-3-ethylurea (EDU) on protection against mortality.**

Treatment, method	Mortality, %
Saline	
Injection	55 ± 7.0
Pump	51 ± 11
EDU	
Injection	
0 h	50 ± 1.0
4 h	50 ± 1.0
24 h	20 ± 1.0 <sup>a</sup>
24 and 4 h before and 24 h after	0.1 ± 0.0 <sup>a</sup>
Pump	18 ± 9 <sup>a</sup>

**NOTE.** Data are mean ± SD of at least 3 separate experiments. Rats received either 0.5 mL of EDU succinate (4 mg/mL) or saline by intramuscular injection at various times before and after challenge, or by osmotic pump loaded with EDU succinate or saline, with a release rate of 0.92  $\mu$ L/h, 24 h before challenge with  $1 \times 10^8$  cfu of *Escherichia coli* and sterile cecal contents.

<sup>a</sup>  $P < .01$ , vs. control.

control group consisting of saline-treated rats was also included. The results (figure 3) demonstrate that, for both groups of rats, splenocytes obtained 24 h after bacterial challenge did not react to in vitro stimulation with the polyclonal mitogen ConA. However, although suppression of T cell proliferation in the EDU-treated rats persisted throughout the entire treatment period, splenocytes obtained from control rats 2 days after challenge proliferated in response to ConA. Seven days after completion of EDU treatment (9 days after bacterial challenge), splenocyte responses to ConA were equivalent in both group of rats (data not shown).

**Effect of EDU in function of T lymphocytes and macrophages in vitro.** In vitro assays were used to confirm the in vivo observations. Splenic T cells stimulated in vitro with ConA and incubated with EDU showed a marked reduction in their ability to release IL-2 and TNF- $\alpha$ , compared with that in untreated cells (figure 4B). However, levels did not reach baseline, as was observed with CsA (used as a positive control; figure 4A). IFN- $\gamma$  concentrations decreased significantly when 10 mmol/L EDU was used. TNF- $\alpha$  production for in vitro-stimulated rat macrophages was significantly reduced by incubation with EDU (figure 4C) in a dose-dependent way.

In addition, incubation of purified T cells with EDU was associated with a concentration-dependent suppression of T lymphocyte proliferation (figure 5). Although the IC<sub>50</sub> for EDU (IC<sub>50</sub>, 4.23 mmol/L) was higher than that for CsA (IC<sub>50</sub>, 0.32  $\mu$ mol/L; used as positive control in this assay), the [<sup>3</sup>H]thymidine incorporation into ConA-stimulated rat T lymphocytes was potently inhibited by increasing concentrations of EDU. This suppression also was observed when murine or human T lymphocytes were

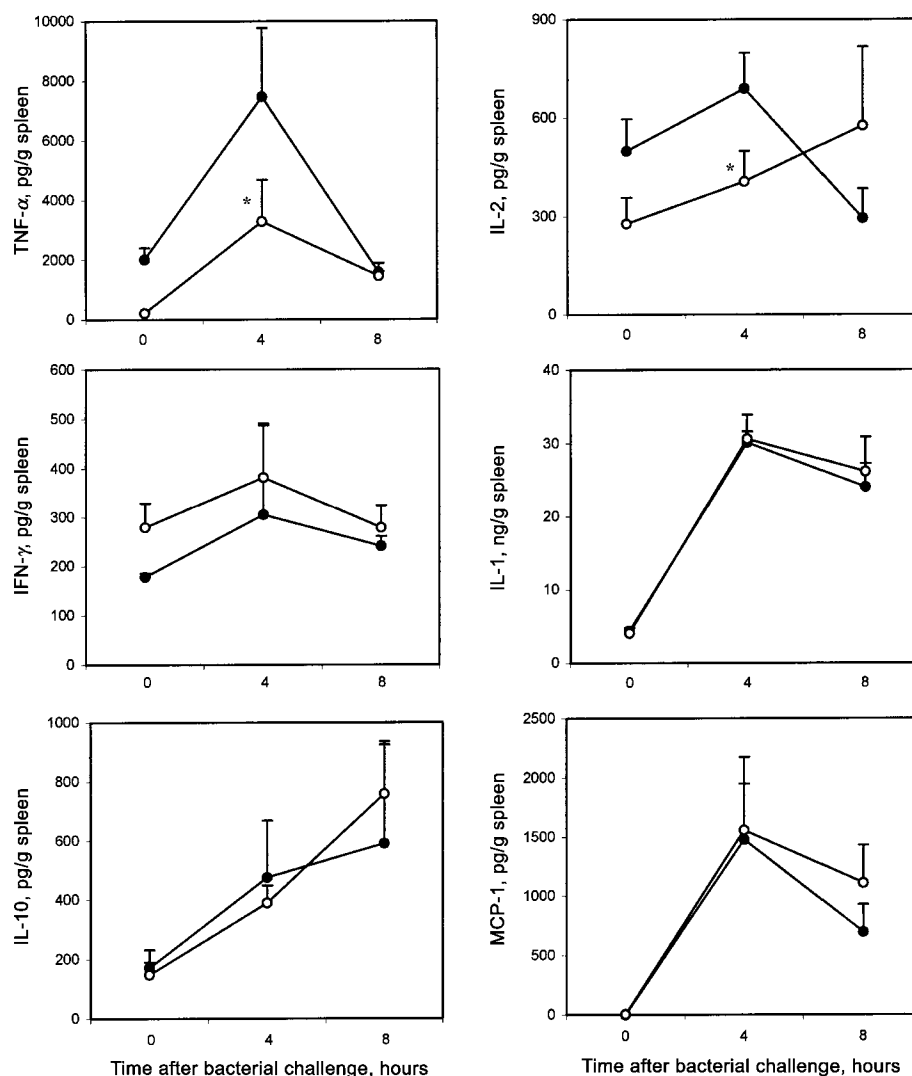
incubated with EDU (data not shown). In all cases, incubation with either CsA or EDU did not affect membrane integrity (i.e., there was no increase in LDH release in cells [data not shown]). Cells appeared morphologically normal, and there was no difference in trypan blue exclusion compared with nontreated cells.

## DISCUSSION

Treatment of patients with severe sepsis still constitutes a clinical challenge. Although some progress has been made in recent years with new antibiotic therapies, the mortality rate for sepsis remains high [17]. Therefore, the use of immunomodulators, agents capable of interacting with the immune system to up- or down-regulate specific aspects of the host response, is appealing. Early trials using  $\beta$ -glucans and *B. fragilis* PS A in the absence of antibiotic therapy showed their effectiveness in preventing intra-abdominal sepsis in the same rat model used for our studies. Administration of these polysaccharides before bacterial challenge yielded a lower rate of mortality than was seen in saline-treated controls [13–15, 18]. Moreover, poly-(1-6)- $\beta$ -glucotri-  
syl-(1-3)- $\beta$ -glucopyranose-glucan has shown some favorable results in preventing postoperative infections after high-risk gastrointestinal noncolorectal procedures in humans [19–21].

These promising early studies with polysaccharides have provoked considerable interest in exploring the possible immunomodulatory effects of other compounds. Our study shows that HA/CMC gels given before challenge with a lethal dose of *E. coli* protect rats against fatal infection in an experimental model of intra-abdominal sepsis. This effect was dose and time dependent, both hallmarks of other anti-infective immunomodulators, and was observed in the absence of any antibiotic treatment.

Since low-molecular-weight HA can be used to prevent allograft rejection and to preserve organ function [22], some researchers would hypothesize that HA is the compound that is responsible for protection in the present experiments. However, assays of components of the gels either alone or in combination revealed no relationship between the presence of HA and protection against lethality. In fact, we found that the protective effect of HA/CMC was due to the nature of the chemical modification imparted by the EDC cross-linking polymer. Under physiologic conditions, the *N*-acylurea groups formed by reaction of either EDC and HA or CMC become hydrolyzed, yielding EDU and free HA or CMC. We showed that treatment with HA/CMC, HA/EDC, CMC/EDC, or purified EDU effectively reduced mortality, which indicates that the polysaccharides had no protective effect and functioned simply as a matrix that progressively released EDU in vivo. Because EDU is a modified urea with low molecular weight (246 g/mol) and is quickly excreted from the body, it is easy to understand that repeated im injections (24 and 4 h before and 24 h after bacterial challenge) offer better protection than does a single in-



**Figure 2.** Effect of 1-(3-dimethylaminopropyl)-3-ethylurea (EDU) on cytokine production after challenge. Rats received 0.5 mL of EDU (○) or saline (●) (4 mg/mL) by intramuscular injection 24 h and 4 h before challenge with  $7 \times 10^7$  cfu of *Escherichia coli* and sterile cecal contents. At 4 and 8 h after challenge, rats were killed, their spleens were removed, and rat-specific enzyme immunoassays for tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-2, monocyte chemotactic protein (MCP)-1, IL-6, IL-1, and IL-10 were performed on spleen extracts ( $n = 5$  rats/group). \* $P < .02$ .

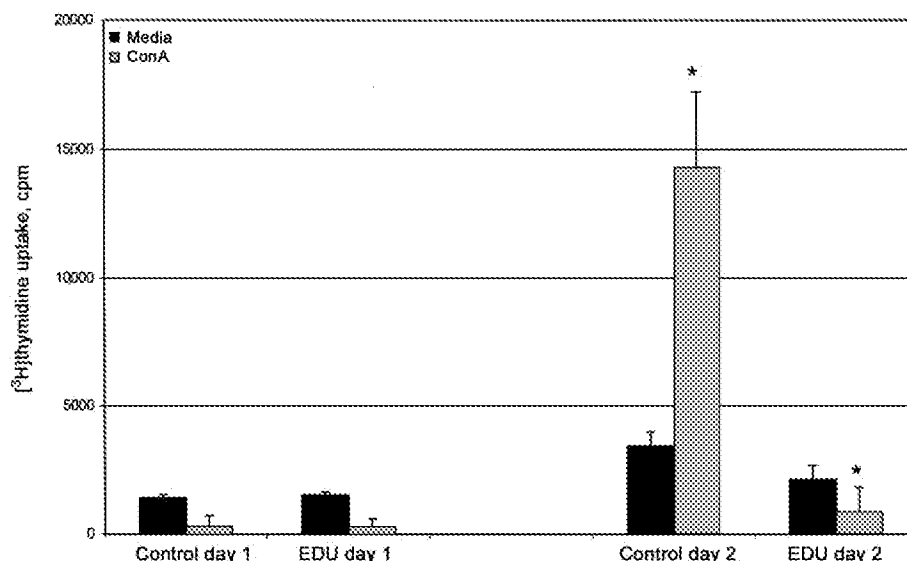
jection 24 h before challenge. Moreover, the significant reduction in mortality by administration of EDU by slow-release pumps, mimicking the progressive liberation of the compound by gels, confirms the importance of EDU bioavailability for protection. We are currently investigating new formulations of EDU (e.g., palmitate EDU) with slower excretion rates.

The rat intra-abdominal sepsis model used in this study constitutes a severe fatal sepsis model. Proinflammatory cytokines (mainly TNF- $\alpha$ , IL-1, and IFN- $\gamma$ ) were rapidly expressed after challenge (within the first 4 h) in response to *E. coli* LPS and other antigens present in the cecal contents; rats died within the first 24 h in response to the overwhelming systemic inflammatory response. Although a correlation between cytokine concentrations in plasma and the outcome of sepsis frequently

has been seen, we measured cytokine levels in spleen extracts, because our previous experience revealed that plasma levels are not always indicative of the actual stage of the infection. Several cytokines exist in membrane-associated forms, and their concentrations within the lymphoid organs are frequently higher than and not correlated with those in the systemic circulation.

To elucidate the mechanism involved in the protective effects seen with EDU, cytokine profiles of treated rats were compared with those from the control rats. Our results show that EDU treatment effectively reduces spleen TNF- $\alpha$  and IL-2 levels in the first hours after challenge. In addition, when EDU was tested in vitro in both purified rat T lymphocytes and peritoneal macrophages, a significant decrease in TNF- $\alpha$  production was observed, compared with that in cells incubated in the absence





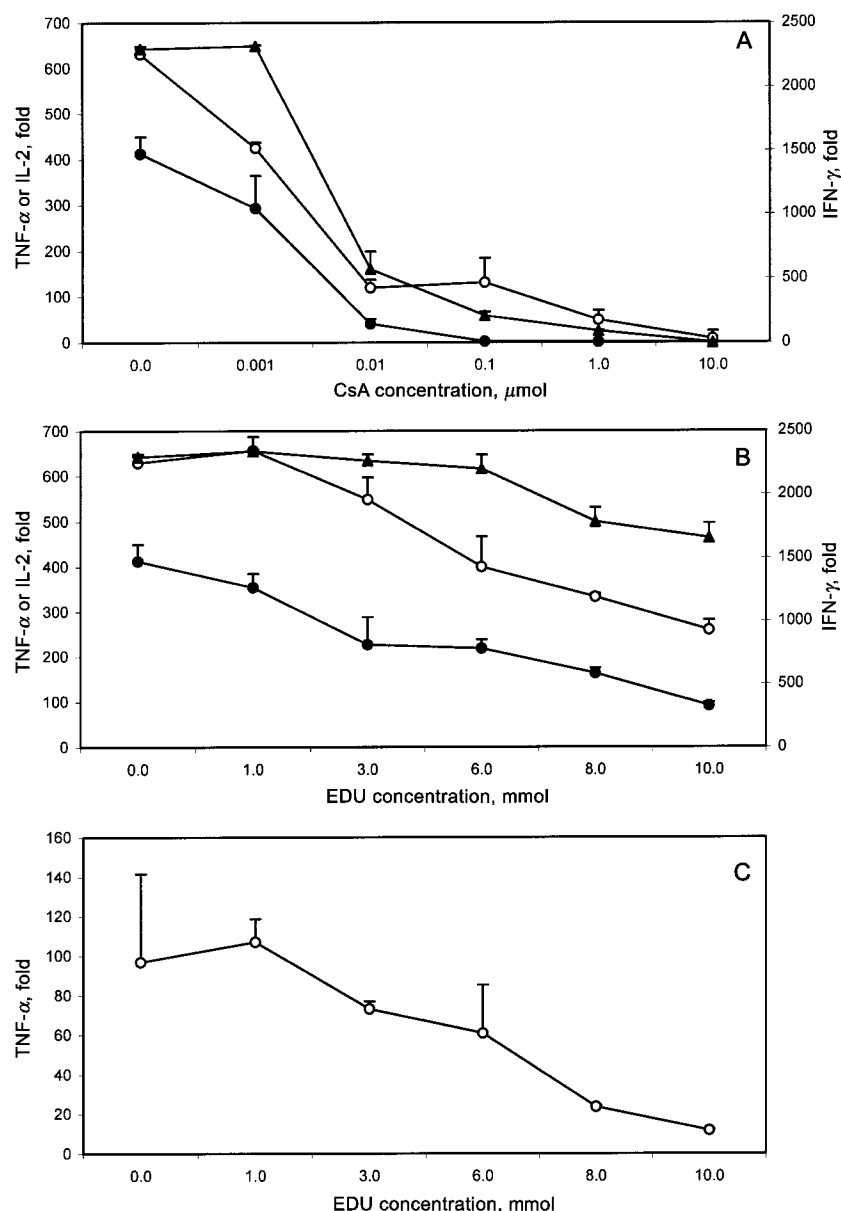
**Figure 3.** Inhibitory effect of 1-(3-dimethylaminopropyl)-3-ethylurea (EDU) on proliferation of T lymphocytes after challenge. Rats received 0.5 mL of EDU or saline (4 mg/mL) by intramuscular injection every 12 h for 2 days before and 2 days after challenge with  $3.5 \times 10^7$  cfu of *Escherichia coli* and sterile cecal contents. At days 1 and 2 after challenge, rats were killed, and their spleens were removed. Splenocytes were then stimulated with 3  $\mu$ g/mL Concanavalin A (ConA) for 4 days. Cultures were pulsed with [ $^3$ H]thymidine for the last 16 h of incubation, and radioactivity was measured ( $n = 5$  rats/group). \* $P < .01$ .

of EDU. TNF- $\alpha$  is the principal mediator of the hemodynamic and inflammatory changes in sepsis that can be fatal [23], so we hypothesize that EDU prevents mortality in our model of severe intra-abdominal sepsis by diminishing the initial proinflammatory response. It is important to note that, although there was no difference in levels of the proinflammatory cytokine IFN- $\gamma$  between control and EDU-treated rats in vivo, we observed a significant decrease in IFN- $\gamma$  production in vitro in ConA-stimulated T lymphocytes after incubation with high concentrations of EDU. Thus, even though our results show that, in the first hours after challenge, EDU decreases TNF- $\alpha$  more effectively than it decreases other proinflammatory cytokines (such as IFN- $\gamma$  and IL-1), concentrations of those cytokines may be reduced in later stages of the infection.

Several authors have described the protective role of anti-inflammatory cytokines (e.g., IL-10 [24–26] or MCP-1 [27]) in animal models of septic inflammatory response. These mediators reduce the production of proinflammatory cytokines, mainly TNF- $\alpha$ , which leads to a reduction in mortality associated with sepsis. However, the decrease in TNF- $\alpha$  levels we observed in EDU-treated rats was not related to increased levels of either IL-10 or MCP-1. Moreover, we found no differences in levels of other Th2-type cytokines, such as IL-6 and IL-4, between control and EDU-treated rats, which suggests that the nature of the protective effect observed with EDU is related to the Th1 response. Recent studies using a murine model of intra-abdominal sepsis have shown that, although enhancement of Th1 response could be related to increased bacterial clearance,

excessive production of Th1 proinflammatory cytokines may be detrimental [28]. Although we did not determine bacterial levels in these experiments, a very interesting observation in our study (confirmed both in vivo and in vitro) is that EDU does not inhibit TNF- $\alpha$  production completely (either by T lymphocytes or macrophages) but maintains attenuated levels that could be adequate for bacterial clearance and are below pathological limits.

EDU effectively reduces IL-2 production both in vivo and in vitro, which suggests that EDU has a direct effect on T cell proliferation. The role of T lymphocytes in intra-abdominal sepsis is still intriguing. Heidecke et al. [29] recently reported defective T cell proliferation and secretion of IL-2 in patients with lethal intra-abdominal infection, compared with that in survivors and healthy control subjects. Marked functional defects in T cells in rats with intra-abdominal sepsis have also been described [30]. These observations suggest the importance of T cells in the long-term immune defense against sepsis mortality. However, our results clearly indicate the role of T cell activation in the deleterious inflammatory response that is observed in the first stages of severe sepsis. We have shown that splenocytes from saline-treated rats had a typical response to in vitro ConA stimulation. Because cells were exposed in vitro to a secondary antigen just 24 h after bacterial challenge, it is easy to understand the lack of response due to T cell anergy [31]. However, 2 days after challenge, control cells were capable of responding to ConA. On the contrary, EDU administration in vivo induces unresponsiveness of splenocytes to in vitro

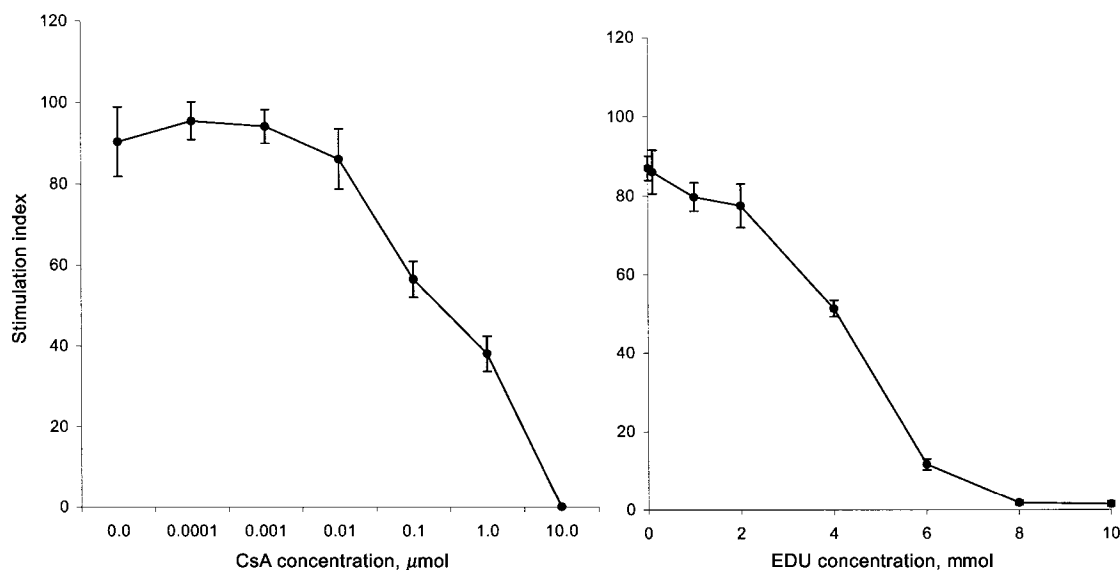


**Figure 4.** Inhibitory effect of 1-(3-dimethylaminopropyl)-3-ethylurea (EDU) on tumor necrosis factor (TNF)- $\alpha$  (○), interleukin (IL)-2 (●), and interferon (IFN)- $\gamma$  (▲) production in concanavalin A (ConA)-stimulated T lymphocytes and lipopolysaccharide (LPS)/IFN- $\gamma$ -stimulated peritoneal macrophages in vitro. T cells were purified from rat splenocytes and incubated with 3  $\mu\text{g/mL}$  ConA for 4 days, in the presence of different concentrations of cyclosporin A (CsA) (A), as control, or EDU (B). C, Macrophages were purified from sterile peritoneal lavage and incubated with 1  $\mu\text{g/mL}$  LPS and 10 ng/mL IFN- $\gamma$  overnight in the presence of different concentrations of EDU. Rat-specific enzyme immunoassays for IL-2, TNF- $\alpha$ , and IFN- $\gamma$  were performed on culture supernatants. Fold concentrations of IL-2, TNF- $\alpha$ , and IFN- $\gamma$  were calculated with cytokine concentrations in supernatants from cells cultured with ConA and EDU or CsA, divided by cytokine concentrations in supernatants from cells cultured with medium alone. Data are mean  $\pm$  SD of at least 3 separate experiments.

ConA stimulation, even after the anergic stage. Furthermore, in vitro treatment of purified T cells with EDU clearly confirmed the suppressive effect of this compound on T cell proliferation, paralleled by down-regulation of IL-2 production.

We now propose that inhibition of T cell activation in the acute phase of the infection accounts for the protection offered by EDU treatment against mortality. This observation, although

novel in this model, is consistent with other studies reporting the importance of T cell proliferation in mortality associated with toxic shock syndrome. Suppression of T cell activation with CTLA4Ig, a fusion protein known to block the costimulatory pathway CD28/B7 that controls T cell response to a variety of antigens, leads to a decrease in mortality in toxic shock syndrome caused by *Staphylococcus aureus* [32]. In addition, blockade with



**Figure 5.** Inhibitory effect of 1-(3-dimethylaminopropyl)-3-ethylurea (EDU) in proliferation of concanavalin A (ConA)-stimulated T lymphocytes in vitro. T cells were purified from rat splenocytes and incubated with 3  $\mu$ g/mL ConA for 2 or 4 days in the presence of different concentrations of EDU or cyclosporin A (CsA) as control. Cultures were pulsed with [ $^3$ H]thymidine for the last 16 h of incubation, and radioactivity was measured. Stimulation index was calculated with the cpm of cells cultured with ConA and EDU or CsA divided by the cpm of cells cultured with medium alone. Data are mean  $\pm$  SD of at least 3 separate experiments.

CTLA4Ig also reduces abscess formation in the rat model for intra-abdominal sepsis used in our study [12].

In conclusion, this work has shown that the protective effect of HA/CMC gels against mortality in the rat intra-abdominal sepsis model actually was conferred by EDU continuously released from the gel complex. EDU has immunomodulatory properties in vivo, not only in suppressing T cell hyperproliferation, but also in maintaining TNF- $\alpha$  and IL-2 levels that seem to be adequate for bacterial clearance but below pathological limits. The accelerated expression pattern of inflammatory cytokines in severe sepsis supports the notion that early immunomodulatory therapy with EDU may play a crucial role in preventing mortality associated with intra-abdominal sepsis. Moreover, EDU may help prevent hyperactive immune responses and T cell-mediated autoimmune disorders.

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